

## ***Interactive comment on “Methanotrophy within the water column of a large meromictic tropical lake (Lake Kivu, East Africa)” by C. Morana et al.***

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First of all, we would like to thank the reviewers for their positive and very constructive comments. In the section below, we will provide a point-by-point reply to the suggestions and comments provided by the reviewers.

Anonymous Referee 2

Reviewer comment 1 : The introduction is too pedestrian. I find no motivation for this particular study in the introduction, neither are new, more recent general and lake-specific knowledge introduced and/or discussed. For instance, the specific types of methanotrophs and their identifying characteristics are not explored at all and the AOM coupled to sulfate reduction is presented as though it was the ONLY process of AOM.

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Recent studies show ANME are able to oxidize methane without the SRB partners. This should be noted in the introduction. Also, the authors should thoroughly discuss previous methane-based studies in Lake Kivu. They should then discuss gaps in research that their study sought to address.

> Reply : The introduction was modified to present more recent literature and provide a better motivation of our work.

Reviewer comment 2 : p. 15666 Line 14 – ‘bur’ should be ‘but’

> Reply : This has been corrected.

Reviewer comment 3 : p. 15667 Line 3 – Which inherent characteristics ? Please list/discuss them.

> Reply : The permanent stratification and the high methane concentration in deep waters of Lake Kivu throughout the year. The revised text now reads : “Because of the permanently stratified nature of its water column and the large amount of CH<sub>4</sub> dissolved in its deep water, the meromictic Lake Kivu offers an ideal natural laboratory to investigate the role of methanotrophy in large tropical lakes.”

Reviewer comment 4 : p. 15667 Line 23 – Is “wanve” supposed to be wave ?

> Reply : Yes, the text has been corrected.

Reviewer comment 5 : p. 15668 Line 19 – What exact volume of headspace was created ?

> Reply : 20 ml. This is now specified in the text.

Reviewer comment 6 : p. 15670 Line 1 – Estimation of reproducibility was based on what ? Please be specific.

> Reply : Based on triplicate measurements on a selection of samples. This is now specified in the text

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Reviewer comment 7 : p. 15670 Line 17 – Here and elsewhere, change “tube” to “tubing”. Specify if PE, PP, PC, PTFE, etc

> Reply : Silicone tubing. Changes have been made in the text.

Reviewer comment 8 : p. 15672 Line 15 – Oxycline seems to start at 40 m rather than 50 m. Please check.

> Reply : To avoid confusion, the text now reads : “CH<sub>4</sub> was abundant in deep waters, with a maximum concentration of 899  $\mu\text{mol L}^{-1}$  at 80 m, however CH<sub>4</sub> decreased abruptly at the bottom of the oxycline, being 4 orders of magnitude lower in surface waters (Figure 1a).”

Reviewer comment 9 : p. 15672 Line 17 – Here and elsewhere, how could you tell where the ‘oxic-ANOXIC transition’ was when the detection limit of your oxygen sensor was as high as 3  $\mu\text{mol L}^{-1}$ ? This means the depths you consider anoxic could actually contain as much as 3  $\mu\text{M}$  oxygen... meaning a much deeper depths could be actually oxic. Are you sure the detection limit was not lower than this? If it was as high as reported, it would be advisable to avoid using the term ‘oxic-anoxic’ or at the least, you should use literature references to support your data in establishing this transitional layer.

> Reply : We agree with the reviewer that using the word “anoxic” might be confusing, and not appropriate given the limit of detection of our oxygen sensor. In the revised version of the manuscript, “oxic-anoxic transition” has been replaced by “transition between oxic and O<sub>2</sub>-depleted waters”. Also, the term “O<sub>2</sub>-depleted waters” is clearly defined in the material and methods as waters where the O<sub>2</sub> concentration was below 3  $\mu\text{mol L}^{-1}$

Reviewer comment 10 : p. 15674 Line 13-15 – The use of ‘oxic-anoxic condition’ and ‘low-oxygen conditions’ is confusing. How can you have transition from oxic to anoxic condition, and then below the anoxic condition, you somehow also have a low-

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oxygen condition? This is an issue because of the detection limit of the oxygen sensor used. However, you can work around this by avoiding using the term “oxic-anoxic transition” > Reply : We agree with the reviewer that using the word “anoxic” might be confusing. See our reply to the previous comment : changes have been made in the text to replace “oxic-anoxic transition” by “transition between oxic and O<sub>2</sub>-depleted waters”. Reviewer comment 11 : p. 15675 Line 19-22 – 105% and 142% are confusing. Please recheck your computations. The microbes cannot oxidize any more methane than what is available. I suggest to either report the average values or use fractionation factors that give reasonable estimates of the fraction of methane that is oxidized. > Reply : The reviewer raised an interesting issue. Actually, Bastviken et al. (2002) reported also values higher than 100 % for the flux of CH<sub>4</sub> oxidized. It is maybe due to the use of linear approximation of the Rayleigh equation, that might not be valid in Lake Kivu because of the high fraction of CH<sub>4</sub> that is oxidized. We revised our calculation using the original equations, and refer now to Coleman et al. (1981). The text now reads : “The fraction of the upward CH<sub>4</sub> flux oxidized within a depth interval can be estimated from a closed-system Rayleigh model of isotope fractionation (Blees et al. 2014) described by the following equation (rearranged from Eq. 11; Coleman et al. 1981):  $\ln(1-f) = \ln((\delta^{13}\text{CH}_4\text{t}+1000)/(\delta^{13}\text{CH}_4\text{b}+1000))/((1/\alpha)-1)$  (8) where f is the fraction of CH<sub>4</sub> oxidized within the depth interval,  $\delta^{13}\text{CH}_4\text{b}$  and  $\delta^{13}\text{CH}_4\text{t}$  are the  $\delta^{13}\text{C}$  values of CH<sub>4</sub> at the bottom and the top of the depth interval, respectively, and  $\alpha$  is the isotope fractionation factor for CH<sub>4</sub> oxidation estimated in Lake Kivu in September 2012 ( $\alpha = 1.016 \pm 0.007$ ). Based on this equation and using a range of isotope fractionation factors (from 1.009 to 1.023), we can estimate that 51-84% of the upward flux of CH<sub>4</sub> was microbially oxidized within a 10 m depth interval in the oxycline (60-70 m) in the Southern Basin during the dry season (September 2012). Similarly, 51-84% of the CH<sub>4</sub> flux was oxidized between 50 m and 55 m in the Northern Basin during the dry season, and 58-89% of the CH<sub>4</sub> flux was oxidized within a wider depth interval (45-70 m) during the rainy season (February 2012). The relatively wide range of the estimated percentage of CH<sub>4</sub> flux oxidized is due to the uncertainty on the isotope fractionation

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factor. Nevertheless, these calculations illustrate clearly the importance of microbial CH<sub>4</sub> oxidation processes in preventing CH<sub>4</sub> to reach the surface waters of the lake”.

Reviewer comment 12 : p. 15676 Line 4-18 – I have issues with the end-members used in the mixing model. The bulk POC should already contain some methanotrophic biomass so it should not be used as the end member of the sedimenting organic matter. Either completely remove these estimates from the paper or you should use C13 of diagnostic biomarkers or better still, use C13 of CO<sub>2</sub>/DIC (and correct for photosynthetic fractionation) as the sedimenting OM end-member.

> Reply : We used the  $\delta^{13}\text{C}$ -POC value in surface waters (5 m) as a sedimenting-OM end-member. This value was -22.9‰ in the Northern Basin during the rainy season, -24.4‰ in the Southern Basin during the dry season, and -23.9‰ in the Northern Basin during the dry season. In a manuscript recently published in Biogeosciences Discussions (Morana et al. 2014), we report that the  $\delta^{13}\text{C}$ -POC values stayed almost constant throughout the year in surface waters ( $-23.8 \pm 0.8\text{‰}$   $n = 19$ ). Similarly, the  $\delta^{13}\text{C}$ -DIC was also rather constant, with values oscillating from +2.4‰ to +3.4‰. Phytoplankton is typically  $\sim 20\text{‰}$  more depleted in  $^{13}\text{C}$  than its source (see Fogel and Cifuentes 1993). Almost 95% of the DIC in surface waters of Lake Kivu is HCO<sub>3</sub><sup>-</sup> (water temperature  $\sim 24^\circ\text{C}$  ; pH  $\sim 9$ ), therefore, using the isotope fractionation between HCO<sub>3</sub><sup>-</sup> and CO<sub>2</sub> reported by Vogel et al. (1970) or Zhang et al. (1995), the  $\delta^{13}\text{C}$  signature of the dissolved CO<sub>2</sub> would approximate  $\sim -5\text{‰}$ . Our  $\delta^{13}\text{C}$ -POC values measured in the surface waters of Lake Kivu are then in a good agreement with an almost exclusive phytoplankton origin of the POC. These data are extensively discussed in Morana et al. (2014). Even if minimal, a contribution of methanotrophic biomass to the POC pool in surface waters would have led to drastically lower  $\delta^{13}\text{C}$ -POC, because of the much lower  $\delta^{13}\text{C}$  values of CH<sub>4</sub> ( $\delta^{13}\text{C}_{\text{CH}_4}$  was never higher than -39 ‰).

Reviewer comment 13 : p. 15679 Line 4 –  $^{13}\text{C}$ -depletion of C16 MUFA was not within the oxycline (Fig 2d). The  $^{13}\text{C}$  depletion rather appears to start right below (or at) the transition between the ‘oxycline’ and the ‘low-oxygen waters’, extending way deeper

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into the ‘the low-oxygen’ depths which could potentially be anoxic depths. This observation should be discussed. Why are the type I methanotrophs active within these depths?

> Reply : This study is not the first to report a strong  $^{13}\text{C}$ -depletion of bacterial lipid markers for aerobic methanotrophic bacteria at the bottom of the oxycline, or in O<sub>2</sub>-depleted waters (see Schubert et al. 2006 for the Black Sea, Brees et al. 2014 for Lake Lugano). This is now discussed in the revised version of the manuscript. See the comment below for a more detailed reply.

Reviewer comment 14 : p. 15679 Line 4 – Also, in the  $^{13}\text{C}$ -labelled methane tracer studies that the authors performed, all the samples that were used for the incubations were taken from depths below the oxycline (except 40 m in Feb 2012) (Fig. 4a & b). In September 2012, the ‘oxycline’ was above 55 m and the ‘low-oxygen waters’ below this depth. The incubated samples were from 62.5 -70 m (Fig 4a). Similarly, in February 2012, the ‘oxycline’ was above 45 m with low oxygen waters below this depth. The samples used for the incubations were from 40m, 50m and 60m (Fig 4b). The incubations that showed labelled  $^{13}\text{C}$ -CH<sub>4</sub> incorporation by C16 MUFA (type I methanotrophs) were all from depths below the oxycline, that is, 65 m in September and 50 m in February. So both the in situ PLFA and tracer PLFA  $^{13}\text{C}$  data show that the type I methanotrophs are active way below the oxycline, in the ‘low-oxygen’ and potentially anoxic depths. The authors should discuss these observations. Based on the data, I am not convinced that the type I methanotrophs are active in methane oxidation in the oxycline or oxic zone as suggested by the authors.

> Reply : This needs to be clarified : all the samples that were used for the incubations were taken from depths below the oxycline (except 40 m in Feb 2012). As mentioned in the caption of the Figure 4., our incubations were performed in the Southern Basin during the dry season (Fig 4a), where oxygen concentrations were higher than 3  $\mu\text{mol L}^{-1}$  down to 65 m (Fig 1a). Therefore, our incubations at 62.5 m and 65 m (fig 4a) were indeed carried out under oxic conditions (at O<sub>2</sub> concentration of 61  $\mu\text{mol L}^{-1}$  and 20

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$\mu\text{mol L}^{-1}$ , respectively). The reviewer's comment that "The incubations that showed labelled  $^{13}\text{C}$ - $\text{CH}_4$  incorporation by C16 MUFA (type I methanotrophs) were all from depths below the oxycline, that is, 65 m in September and 50 m in February": might arise from a misreading and inversion of the profile obtained from the Southern Basin (Fig1a) and the Northern Basin (Fig1b, where the water column was oxic down to 55 m). The  $^{13}\text{C}$ - $\text{CH}_4$  incorporated in C16 MUFA at 65 m in the Southern Basin (September 2012) was fixed in presence of oxygen, and a substantial amount of tracer was also incorporated in C16 MUFA under oxic conditions in the Northern Basin (February 2012), at 40 m (Fig 4b). To us, the fact that C16 MUFA, not C18 MUFA, were labelled during our incubation under oxic conditions unambiguously indicates that type I methanotrophs were active in methane oxidation at the bottom of the oxycline, and at the transition between oxic and  $\text{O}_2$ -depleted waters. It is however correct that some  $^{13}\text{C}$  was incorporated in C16 MUFA at 50 m (5 m below the bottom of the oxycline) in February 2012, in the Northern Basin. Due to the limit of detection of our oxygen sensor, it is difficult to know if the waters at 50 m was truly anoxic, or micro-oxic, with  $\text{O}_2$  concentration lower than  $3 \mu\text{mol L}^{-1}$  but still sufficiently high to support some aerobic  $\text{CH}_4$  oxidation. In a recently published paper, Blee et al. (2014) provided multiple lines of evidence for micro-aerobic methane oxidation, 40 m below the chemocline of Lake Lugano. They measured aerobic methane oxidation at nanomolar  $\text{O}_2$  concentration, well below the limit of detection of our oxygen sensor. We revised our text and extended our discussion on this aspect. The revised text now reads : "Nevertheless, in February 2012 the C16 MUFA appeared to be strongly depleted in  $^{13}\text{C}$  below the transition between oxic and  $\text{O}_2$ -depleted waters (Figure 2d). This study is not the first to report strong  $^{13}\text{C}$ -depletion of bacterial lipid markers for aerobic methanotrophic bacteria in  $\text{O}_2$ -depleted waters (see Schubert et al. 2006 for the Black Sea, Blee et al. 2014 for Lake Lugano). The presence of methanotrophic bacterial biomass below the oxycline could simply result from gravity-driven physical particle transport from oxic waters, but it has been also demonstrated that some aerobic methanotrophs are able to persist under low oxygen conditions in a reversible state of reduced metabolic

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activity (Roslev & King 1995). By contrast, the recovery of these aerobic methanotrophs after  $\text{CH}_4$  deprivation under oxic conditions is less successful because they have been found to degrade a significant amount of cell proteins (Roslev & King 1995). Blee et al. (2014) suggested that this physiological preference for  $\text{O}_2$  starvation than  $\text{CH}_4$  starvation under oxic conditions would drive aerobic methanotrophs toward the  $\text{O}_2$ -depleted part of the oxygen continuum. This concept seems particularly important in tropical lakes because the thermal stratification of the water column is usually very dynamic in these systems due to the small temperature gradient, allowing episodic, yet frequent,  $\text{O}_2$  intrusion into deeper waters. Aerobic methanotrophs in dormancy would recover quickly after the episodic  $\text{O}_2$  injection, and resume rapidly micro aerobic  $\text{CH}_4$  oxidation (Blee et al. 2014)".

Reviewer comment 15 : p. 15680 Line 2 – While previous studies have also noted the involvement of sulfate reducing bacteria in the deep waters of the lake, as was observed in this study, 10 Me 16:0 and C17 MUFA are not typically used as biomarkers for sulfate-reducing bacteria. The use of these biomarkers should be thoroughly and convincingly discussed in the context of wider literature.

> Reply : Due to the low phylogenetic resolution of PLFA analyses, we acknowledge that the labelling of 10me16:0 and C17 MUFA does not necessarily reflect a coupling between  $\text{SO}_4^{2-}$  reduction and anaerobic methane oxidation. This issue was also raised by reviewer 1, see our reply to his/her comment. The discussion now reads : "A significant MBP rate ( $1.3 \mu\text{mol L}^{-1} \text{d}^{-1}$ ) was measured under low-oxygen conditions ( $< 3 \mu\text{mol L}^{-1}$ ) at 60 m during the rainy season (February 2012). Moreover, the PLFA labelling pattern was drastically different, with a more important specific  $^{13}\text{C}$  incorporation into 10Me16:0 and C17 MUFA instead of the C16 MUFA, relative to their concentrations. This different labelling pattern suggests that a different population of methanotrophs was active in  $\text{CH}_4$  oxidation deeper in the water column. Archaea lack ester-linked fatty acids in their membrane and are therefore undetectable in PLFA analysis. However 10Me16:0 and C17 MUFA are known to be especially

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abundant in sulphate-reducing bacteria (Macalady et al. 2000, Boschker and Middelburg 2002), one of the syntrophic partner of anaerobic CH<sub>4</sub> oxidizing archaea (Knittel and Boetius 2009). Hence, the specific labelling of 10Me16:0 and C17 MUFA under low-oxygen conditions could indicate that a fraction of the upward flux of CH<sub>4</sub> was oxidized syntrophically by an archaea/bacteria consortium, and might support the hypothesis that the bacterial partner grows on CH<sub>4</sub>-derived carbon source supplied by anaerobic methane oxidizers within the consortium, as already suggested by the results of an *in vitro* labelling (<sup>13</sup>CH<sub>4</sub>) study (Blumenberg et al. 2005). However, our data does not necessarily imply that anaerobic methane oxidation would be coupled with SO<sub>4</sub><sup>2-</sup> reduction, as some sulphate-reducing bacteria have been also found to be able to reduce iron (Coleman et al. 1993). Furthermore, the phylogenetic resolution of SIP-PLFA analyses is rather low (Uhlík et al. 2009), and recent studies showed that anaerobic methane oxidation could be carried out syntrophically by consortium between methanotrophic archaea and denitrifying bacteria (Raghoebarsing et al. 2005), or between methanotrophic archaea and manganese reducing bacteria (Beal et al. 2009). Further investigations would be needed to address more accurately which is the electron acceptors coupled to anaerobic CH<sub>4</sub> oxidation”.

Reviewer comment 16 : Please include a map of the Lake showing the study sites.

> Reply : Figure was added as suggested.

Reviewer comment 17 : Fig 1 (a,b,c) : Please use different scales for the <sup>13</sup>C of CH<sub>4</sub> and <sup>13</sup>C of POC. Also, the CH<sub>4</sub> concentrations should be reported in mmol L<sup>-1</sup>. I reckon that using the same unit as oxygen allows for easy comparison, but in this particular instance, it is better to keep the methane concentration in mmol L<sup>-1</sup>. With this figure and all others, it would be informative to the reader to indicate the precision of the measurements by way of error bars.

> Reply: Units were changed as suggested

Reviewer comment 18: Fig 3. It will help the reader if you include the time (hours) on

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each data point.

> Reply : Figure was changed as suggested.

Reviewer comment 19 : Fig 4. You should note in the figure title that all the samples in Fig 4a that were incubated were from depths below the oxycline and within the low-oxygen region. Similarly, include in the title that 50 m and 60 m are below the oxycline, and are within the low oxygen depths.

> Reply : Only the samples “67.5 m” and “70 m” of the Fig 4a and the samples “50 m” and “60 m” from the Fig 4b were incubated under low oxygen conditions (> 3 μmol L<sup>-1</sup>), as explained above. The caption of the Figure 4 now reads : “Figure 4. Specific CH<sub>4</sub>-derived C incorporation pattern into phospholipid fatty acids (PLFA) (incorporation rates of C into PLFA normalized on PLFA concentration, d<sup>-1</sup>) in (a) September 2012 (dry season) in the Southern Basin and (b) in February 2012 (rainy season) in the Northern Basin. Dissolved oxygen concentration was lower than 3 μmol L<sup>-1</sup> at 67.5 m and 70 m (a), and 50 m and 60 m (b).

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